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Effects of conformational selectivity and of overlapping kinetically influential ionizations on the characteristics of pH-dependent enzyme kinetics

Implications of free-enzyme pK_a variability in reactions of papain for its catalytic mechanism

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1. The effects of selection by a small molecule, when binding to a protein, of a particular conformation from an equilibrium stereopopulation on the characteristics of the pH-dependence of reaction with a reactivity probe or substrate were determined by analysis of an appropriate kinetic model. For reaction in one protonic state containing an equilibrium mixture of two conformational isomers, the pH–second-order rate constant (k) profile is of conventional sigmoidal form. The apparent pK_a value is a composite of the pK_a values of the two conformational states. The value of $pK_{app.}$ for a given enzyme under given experimental conditions will always be the same (provided that the site-specificity assumed in the model is maintained) irrespective of whether only one conformation reacts or both react, with the same or with different rate constants. The experimentally determined pH-independent rate constant ($\tilde{k}_{app.}$) is an average of the reactivities of the two conformational states weighted in favour of the predominant form. The presence of an additional but unreactive conformational state also affects the value of $\tilde{k}_{app.}$. 2. The possibility that overlapping acid dissociations that affect the reactivity of the enzyme might provide pH–k profiles often indistinguishable in practice from simple sigmoidal dissociation curves and subject to variability in apparent pK_a values was evaluated by a simulation study. If two reactive protonic states of the enzyme respond differently to changes in the structure of the substrate or site-specific reactivity probe, differences in apparent pK_a values of up to approx. 1 unit can be exhibited without deviation from sigmoidal behaviour being reliably observed. 3. Differences in apparent pK_a values observed in some site-specific reactions of papain and their possible consequences for its catalytic mechanism are discussed.

The possibility that a small molecule, such as a substrate or reactivity probe, when binding to a protein, might select a particular conformation of the protein from an equilibrium stereopopulation has important implications for enzyme mechanisms. This phenomenon has been called 'fluctuation fit' by Straub & Szabolcsi (1964; cited by Polgár & Halász, 1978). The further possibility that this type of conformational selectivity might be detectable by study of pH-dependent kinetics as suggested by Polgár & Halász (1978) merits careful consideration. Conformational selectivity might be particularly relevant in connection with the study of cysteine proteinases in view of suggestions that the catalytic sites of these enzymes might be capable of substantial conformational mobility involving the relative dispositions of the thiol and imidazole groups of the catalytic site (Drenth *et al.*, 1976; Brocklehurst & Malthouse, 1978, 1980; Angelides & Fink, 1978, 1979*a*,*b*; Brocklehurst *et al.*, 1979*a*,*b*). For example, thiol-specific probes with protonated leaving groups such as 2-pyridyl disulphides at low pH (Brocklehurst, 1982) might be expected to react with the thiolate anion of an ion-pair conformation in which the imidazolium ion is as far away as possible from the protonated mercaptopyridine leaving group to minimize electrostatic repulsion. By contrast, some reactivity probes, such as anionic alkylating agents (Wallenfels & Eisele, 1968; Chaiken & Smith, 1969*a*,*b*; Jolley &

Yankeelov, 1972; Polgár & Halász, 1978; Brocklehurst *et al.*, 1982), benzofuroxan (Shipton & Brocklehurst, 1977) and unprotonated 2-pyridyl disulphides with hydrophobic side chains that might bind in or near the S_2-subsite of papain (Brocklehurst *et al.*, 1979*a*; Patel & Brocklehurst, 1982), appear to react with the thiolate anion of the ion-pair in a conformation that allows simultaneous interaction of the imidazolium ion of the ion-pair with another part of the probe reagent. This is the type of conformation that is commonly supposed to permit acylation of these enzymes by substrate, as evidenced by the bell-shaped profiles of plots of pH versus $k_{cat.}/K_m$ with pK_I approx. 4 and pK_{II} approx. 9 (see, e.g., Brocklehurst *et al.*, 1981; Polgár & Halász, 1982).

It was therefore of considerable interest that Polgár & Halász (1978) suggested that, if different substrates or probe reagents select, for reaction with a given functional group, different conformations of an enzyme from an equilibrium stereopopulation, (*a*) a simple pH–rate profile might not necessarily be obtained and (*b*) different apparent pK_a values characteristic of the free enzyme might be exhibited. If these suggestions could be shown to be correct, this would have important consequences for mechanistic study of enzymes. In the present paper, however, analysis of a minimal kinetic model of conformational selectivity demonstrates both the simple sigmoidal form of pH-dependence that is usual when one ionizing group is considered, and, for reactions with low rate constants where quasi-equilibrium would be predicted, that the same apparent pK_a value characteristic of the free enzyme will always be obtained whether or not several rapidly fluctuant conformations react or whether different reagents react selectively with different conformations.

A mechanism by which different apparent pK_a values characteristic of the free enzyme molecule can be exhibited in its reactions with different substrates or reactivity probes is validated by a computer simulation study. This mechanism, which involves ionizations with overlapping pK_a values, could account for variation in the apparent pK_a values of cysteine proteinases such as papain. With error-free data, variation in pK_a up to approx. 1 unit can be obtained without convincing distortion of the simple ionization curve. With real scattered data, somewhat greater variations might be found without distortion being reliably detected. Distortion of sigmoidal ionization curves by co-operative ionizations has been discussed by Bendall & Lowe (1976), in terms of microscopic pK_a values, in connection with the pH-dependent fluorescence of papain inhibited by reaction with 3-bromo-1,1,1-trifluoropropanone. Evidence for co-operative ionizations in the free papain molecule has been presented previously (see Brocklehurst, 1982, and references cited therein). The phenomenon discussed in the present paper, in which overlapping ionizations can provide for apparent pK_a variation without appreciable profile distortion together with the variations in experimental pK_a values reported by Polgár & Halász (1978), seems to support the suggestions (Shipton & Brocklehurst, 1978; Allen *et al.*, 1978; Jarvis & Brocklehurst, 1982) that the catalytic activity of papain in acidic media might be modulated by ionizations additional to those of the thiol group and the imidazolium ion.

Methods

Error-free values of the second-order rate constant, k, were generated by using eqn. (13) (see the Results and discussion section), various values of its characterizing parameters and a PDP 11/23 computer for pH values between 2.6 and 7.0 at intervals of 0.2 pH unit. These pH–k data were then used to provide the values of $pK_{app.}$ and $\tilde{k}_{app.}$ characteristic of a simple sigmoidal profile by fitting the data to eqn. (8) by use of the optimization procedure described previously (Baines & Brocklehurst, 1982). These parameters were used to generate curves corresponding to eqn. (8) (see Fig. 1). To examine the properties of eqn. (13) at low pH plotted in logarithmic form (Fig. 2), some data were generated also in the range down to pH 0.

Results and discussion

Consequences of conformational selectivity for pH-dependent kinetics

The consequences of conformational selectivity for the shape and characteristics of a pH–rate profile may be assessed by analysis of the simple model in Scheme 1.

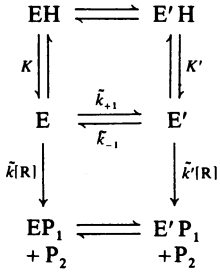
In terms of Scheme 1 the rate of formation of the common product, P_2, by the two apparent second-order reactions shown can be expressed in terms of total concentrations and the pH-dependent second-order rate constant, k (eqn. 1), or in terms of specific conformers E and E′ of the reactive proton-deficient state and the pH-independent rate constants $\tilde{k}$ and $\tilde{k}'$ (eqn. 2):

$$d[P_2]/dt = k[E]_T[R] \quad (1)$$

$$d[P_2]/dt = (\tilde{k}[E] + \tilde{k}'[E'])[R] \quad (2)$$

Combination of eqns. (1) and (2) with the conservation equation (eqn. 3), the acid dissociation constants (eqns. 4 and 5) and the equilibrium conformer ratio (eqn. 6) provides the expression (eqn. 7) for the pH-dependent rate constant, k:

$$[E]_T = [EH] + [E'H] + [E] + [E'] \quad (3)$$



Scheme 1. *Kinetic model for the reaction of a site-specific reactivity probe (R) with the base forms (E and E') of two conformers of an enzyme to provide enzyme derivatives EP_1 and $E'P_1$ and the common product P_2*

EH and E'H are the corresponding acidic forms of the two conformers; K and K' are the two acid dissociation constants; $\tilde{k}_{+1}$ and $\tilde{k}_{-1}$ are pH-independent first-order rate constants; $\tilde{k}$ and $\tilde{k}'$ are the two pH-independent second-order rate constants for the reactions of R with E and E' respectively. The reaction is considered to obey second-order kinetics (first-order in both enzyme and R), so that, if ER and E'R adsorptive complexes form, [R] is considered to be much less than the relevant dissociation constants. An analogous model in which E and E' replace EP_1 and $E'P_1$ can be formulated for a catalysis. For this model $\tilde{k}$ and $\tilde{k}'$ would be $\tilde{k}_{cat.}/\tilde{K}_m$ and $\tilde{k}'_{cat.}/\tilde{K}'_m$. Charges and unbound protons are not shown.

$$K = \frac{[E][H^+]}{[EH]} \quad (4)$$

$$K' = \frac{[E'][H^+]}{[E'H]} \quad (5)$$

$$\alpha = \frac{[E']}{[E]} = \frac{\tilde{k}_{+1}}{\tilde{k}_{-1}} \quad (6)$$

$$k = \frac{(\tilde{k} + \alpha\tilde{k}')/(1+\alpha)}{1 + \dfrac{[H^+]}{(1+\alpha)KK'/(\alpha K + K')}} \quad (7)$$

Eqn. (7) is of the form given in eqn. (8), which is the usual equation that characterizes reaction of the base form of a monobasic acid in terms of the pH-independent rate constant $\tilde{k}_{app.}$ and the apparent pK_a (p$K_{app.}$) (eqns. 9 and 10):

$$k = \frac{\tilde{k}_{app.}}{1 + \dfrac{[H^+]}{K_{app.}}} \quad (8)$$

$$\tilde{k}_{app.} = (\tilde{k} + \alpha\tilde{k}')/(1+\alpha) \quad (9)$$

$$pK_{app.} = -\log[(1+\alpha)KK'/(\alpha K + K')] \quad (10)$$

The form of eqns. (7) and (8) predicts a conventional sigmoidal acid dissociation curve for the pH–k profile. Furthermore this will always be characterized by the same value of p$K_{app.}$ for a given enzyme under given experimental conditions (eqn. 10) irrespective of the nature of the reagent or substrate (provided that site-specificity, assumed in the model, is maintained). This conclusion applies irrespective of whether only one conformation reacts or both react, with the same or with different rate constants. It is clear from eqn. (10) that the apparent pK_a characteristic of the enzyme molecule is a composite of the pK_a values of the two conformational states, the contribution of each being controlled by the equilibrium conformer ratio, α. For example, if $\alpha \ll 1$ (so that E predominates over E') and $\alpha K \ll K'$, p$K_{app.}$ approximates closely to pK, i.e. the pK_a value characteristic of the predominant conformational state.

Eqn. (9) shows that the presence of an additional conformational state of the enzyme can affect the experimentally determined reactivity (characterized by $\tilde{k}_{app.}$). The value of $\tilde{k}_{app.}$ is an average of the reactivities of the two conformational states weighted in favour of the reactivity of the predominant form. The presence of an additional but unreactive conformational state (Scheme 1 with either $\tilde{k}$ or $\tilde{k}'$ set equal to zero) also affects the observed reactivity, since eqn. (9) becomes either eqn. (11) or eqn. (12):

$$\tilde{k}_{app.} = \tilde{k}/(1+\alpha) \quad (11)$$

$$\tilde{k}_{app.} = \alpha\tilde{k}'/(1+\alpha) \quad (12)$$

Variation in kinetically determined pK_a values can arise from non-equilibrium conditions (Brocklehurst & Dixon, 1976, 1977), but it is not necessary then to assume additionally a duality or multiplicity of reactive conformational states to account for the variation.

Consequences of overlapping ionizations for pH-dependent kinetics

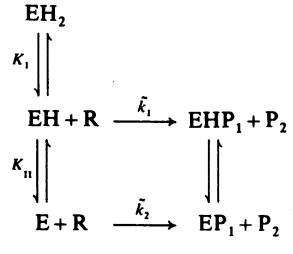
The failure of conformational selectivity to provide a mechanism by which kinetically determined apparent pK_a values might vary with the structure of the substrate or probe reagent led us to consider other mechanisms that might account for such variation. Polgár & Halász (1978) discussed most of the obvious possibilities and pointed out that these would be unlikely to apply to reactions of truly site-specific reagents of relatively low reactivity, where quasi-equilibrium conditions would be expected to obtain.

One possible mechanism that had not been adequately evaluated involves the possibility that overlapping ionizations that affect the reactivity of the enzyme might provide pH–k profiles indis-

tinguishable in practice from simple sigmoidal ionization curves. The apparent pK_a values of such curves might then be subject to variability if two reactive protonic states of the enzyme respond differently to changes in the structure of the substrate or site-specific reactivity probe. To evaluate this mechanism, pH–k data were generated by using eqn. (13) and these data were then fitted to eqn. (8) by using the optimization procedure referred to in the Methods section:

$$k = \frac{\tilde{k}_1}{1 + \frac{[H^+]}{K_I} + \frac{K_{II}}{[H^+]}} + \frac{\tilde{k}_2}{1 + \frac{[H^+]^2}{K_I K_{II}} + \frac{[H^+]}{K_{III}}} \quad (13)$$

Eqn. (13) describes the pH-dependence of the second-order rate constant, k, for formation of the common product, P_2, by the two protonic states of the model shown in Scheme 2 [see Brocklehurst (1974) for discussion of an extended model and rate equation of which eqn. (13) is a truncated form]. Eqn. (8) describes the pH-dependence of k for product formation by a proton-deficient state formed by a single protonic dissociation and takes the usual form of a simple sigmoidal pH–k profile. The extent to which experimental data appropriate to eqn. (13) might appear to be fitted adequately by the simpler equation, eqn. (8), depends on the separation of pK_I and pK_{II}, the relative values of $\tilde{k}_1$ and $\tilde{k}_2$, and the quality and extent of the experimental data. Fig. 1(*a*) shows that, when pK_I and pK_{II} are separated by 1.5 units, deviation from simple sigmoidal behaviour should be readily detected, except possibly when $\tilde{k}_1$ approximates closely either to zero or to the value of $\tilde{k}_2$. When pK_I and pK_{II} are separated by up to 1 unit (Figs. 1*b* and 1*c*), data appropriate to Scheme 2 and eqn. (13) could appear to fit a simple single ionization profile (eqn. 8) for almost all values of $\tilde{k}_1$ and $\tilde{k}_2$. Furthermore, for a given separation of pK_I and pK_{II}, the value of $pK_{app.}$ (eqn. 8) decreases as $\tilde{k}_1$ increases relative to $\tilde{k}_2$. The variation in $pK_{app.}$ with variation in the relative values of $\tilde{k}_1$ and $\tilde{k}_2$ is illustrated in Table 1. The phenomenon of variability of $pK_{app.}$ with a good degree of retention of sigmoidal shape is illustrated in Fig. 1 with the use of error-free data. The small deviations from simple sigmoidal behaviour in some of the plots might well be obscured when real, scattered, data are plotted.



Scheme 2. *Kinetic model for the reaction of a site-specific reactivity probe (R) with two protonic states (EH and E) of an enzyme to produce the enzyme derivative ($EHP_1 \rightleftharpoons EP_1$) and the common product P_2*

The pH-dependence of the experimentally determined second-order rate constant, k, is a function of the molecular (macroscopic) pK_a values of the free enzyme, pK_I and pK_{II}, and of the pH-independent rate constants, k_1 and k_2 [see eqn. (13) in the text]. This dependence of k on free-enzyme pK_a values is predicted also from more elaborate models showing explicitly the intermediary adsorptive complex reversibly connected by a thermodynamic box when, as would be the case for most reactivity probes and also for relatively slow substrates, the quasi-equilibrium condition holds (see, e.g., Brocklehurst, 1982). For the analogous substrate model, $\tilde{k}_1$ and $\tilde{k}_2$ would be pH-independent values of $k_{cat.}/K_m$, and EHP_1 and EP_1 would be free enzyme forms. Charges and free protons are not shown.

Table 1. *Variation in apparent pK_a value caused by reactivity differences between two protonic states of an enzyme with overlapping ionizations*

The values of pK_I, pK_{II}, $\tilde{k}_1$ and $\tilde{k}_2$ were used to generate pH–k data pairs by using eqn. (13) for pH values in the range 2.6–7.0 at intervals of 0.2 pH unit. The values of $pK_{app.}$ and $\tilde{k}_{app.}$ are the characterizing parameters of eqn. (8) obtained by using these data and an optimization procedure. For all the values of $\tilde{k}_1$ shown (except $\tilde{k}_1 = 0$) plots of $\log k$ versus pH contain a line of slope +1 at low pH, and thus these plots provide no evidence for a pH-dependence any more complex than eqn. (8).

pK_I	pK_{II}	$\tilde{k}_1$	$\tilde{k}_2$	$pK_{app.}$	$\tilde{k}_{app.}$
4.0	4.5	1.0	1.0	3.92 ± 0.01	1.05 ± 0.01
		0.9		3.98 ± 0.01	1.03 ± 0.005
		0.8		4.03 ± 0.01	1.02 ± 0.003
		0.7		4.11 ± 0.005	1.01 ± 0.002
		0.6		4.18 ± 0.004	1.01 ± 0.002
		0.5		4.26 ± 0.003	1.00 ± 0.001
		0.4		4.34 ± 0.004	1.01 ± 0.002
		0.3		4.42 ± 0.005	1.01 ± 0.002
		0.2		4.49 ± 0.008	1.01 ± 0.003
		0.1		4.57 ± 0.01	1.01 ± 0.005
		0		4.65 ± 0.02	1.02 ± 0.007
4.0	5.0	1.0	1.0	3.98 ± 0.01	1.03 ± 0.003
		0.9		4.04 ± 0.001	1.00 ± 0.0003
		0.8		4.13 ± 0.005	0.99 ± 0.002
		0.7		4.23 ± 0.01	0.98 ± 0.004
		0.6		4.34 ± 0.01	0.98 ± 0.005
		0.5		4.46 ± 0.015	0.97 ± 0.006
		0.4		4.59 ± 0.02	0.98 ± 0.006
		0.3		4.72 ± 0.01	0.98 ± 0.005
		0.2		4.85 ± 0.006	0.99 ± 0.003
		0.1		4.96 ± 0.01	1.01 ± 0.006
		0		5.07 ± 0.01	1.01 ± 0.006

The circumstances in which deviations of the data from a simple sigmoidal curve at pH values below the apparent pK_a value might be revealed convincingly by logarithmic plots are illustrated in Fig. 2. Negative deviations of the data from a line of slope +1 will appear when the squared term in the denominator of eqn. (13) makes a significant contribution to the value of k. The extreme case is when $\tilde{k}_1 = 0$, which provides for a slope of +2 in the logarithmic plot at low pH. It is instructive that when $\tilde{k}_1 = 0.1$ (i.e. 10% of $\tilde{k}_2$) a line of slope +1 is still obtained and that $\tilde{k}_1$ needs to be a very small fraction indeed of $\tilde{k}_2$ before significant deviation of the line from a slope of +1 is seen. For non-zero values of $\tilde{k}_1$, the line reverts to a slope of +1 at very low pH, when the squared term in eqn. (13) again has negligible effect on the value of k. In many cases, particularly when $pK_{app.}$ is in the range 3–5, it will

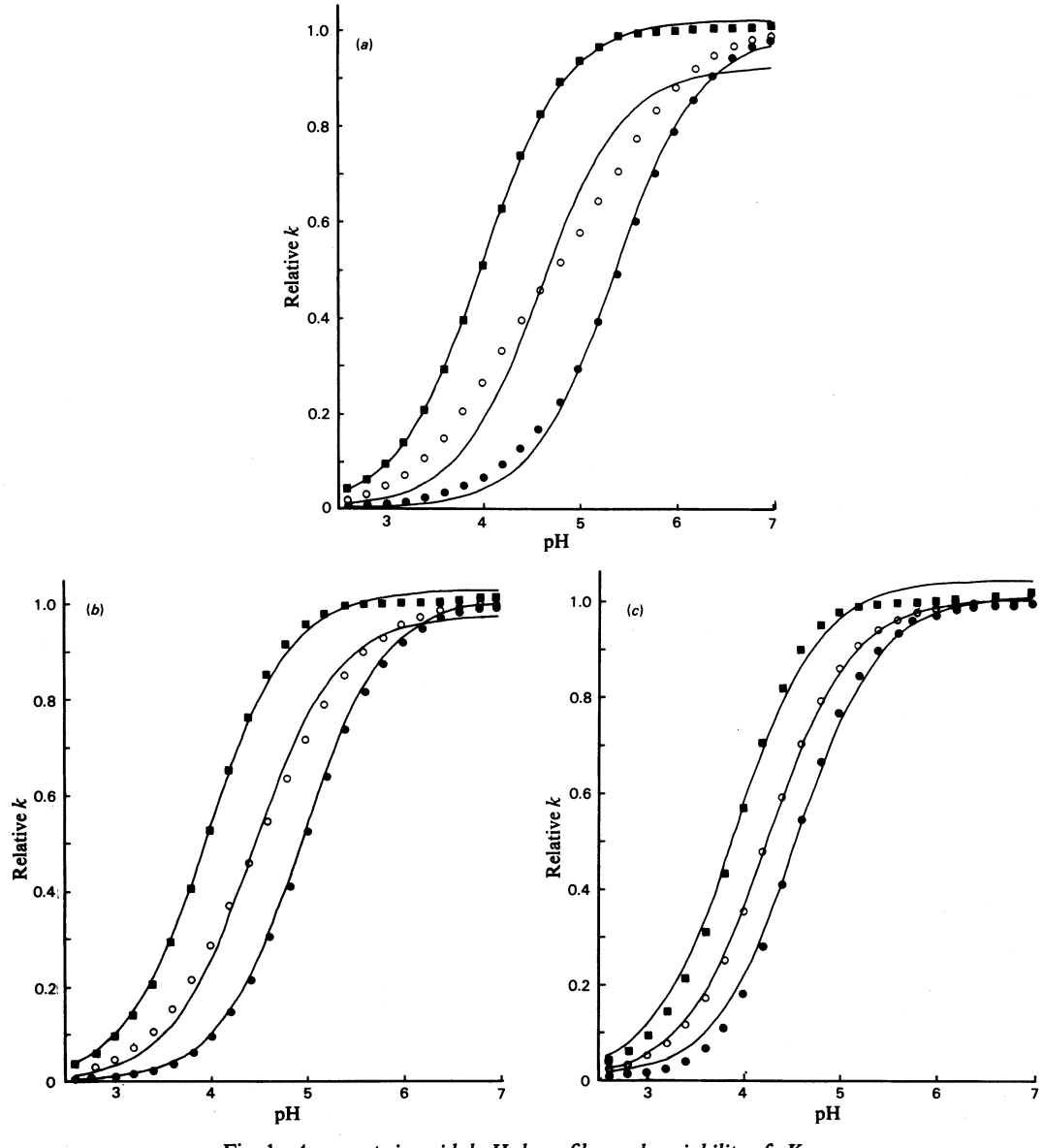


Fig. 1. *Apparent sigmoidal pH–k profiles and variability of $pK_{app.}$*
The data points relate to eqn. (13) of the text for $\tilde{k}_2 = 1.0$ and for $\tilde{k}_1 = 0.1$ (●), $\tilde{k}_1 = 0.5$ (○) and $\tilde{k}_1 = 1.0$ (■): (*a*) $pK_I = 4.0$, $pK_{II} = 5.5$; (*b*) $pK_I = 4.0$, $pK_{II} = 5.0$; (*c*) $pK_I = 4.0$, $pK_{II} = 4.5$. The continuous lines are the best-fit simple sigmoidal curves obtained by fitting the data points to eqn. (8) of the text by the optimization procedure referred to in the Methods section.

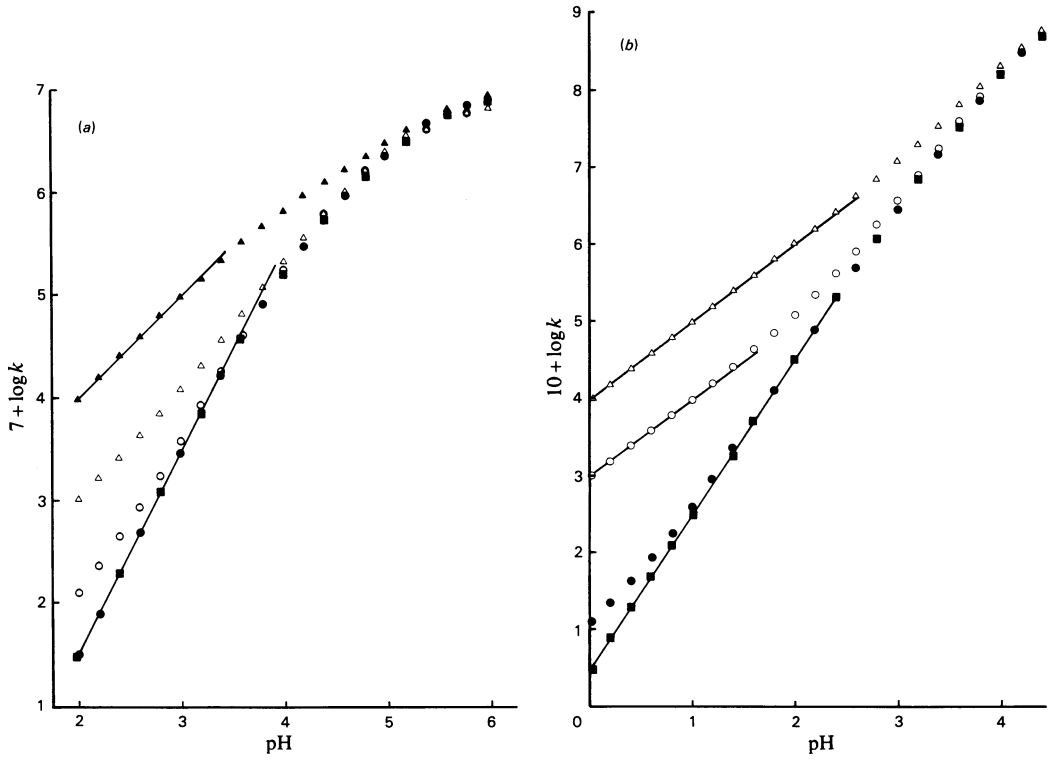


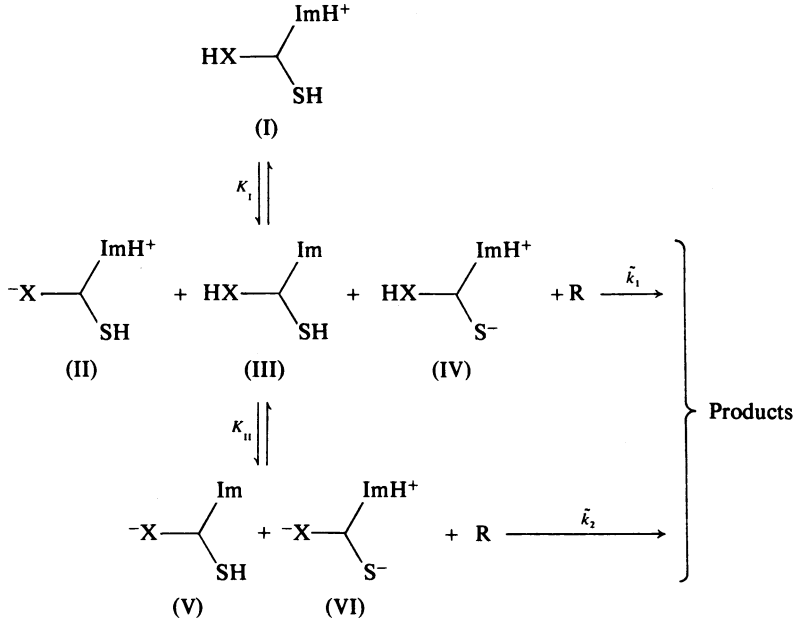
Fig. 2. *Logarithmic plots of pH–k data corresponding to eqn. (13) and* $\tilde{k}_2 = 1.0, pK_I = 4.0, pK_{II} = 5.5$
(*a*) pH range down to 2.0; ▲, $\tilde{k}_1 = 0.1$ (the continuous line is of slope +1); △, $\tilde{k}_1 = 0.01$; ○, $\tilde{k}_1 = 0.001$; ●, $\tilde{k}_1 = 0.00001$ (the continuous line is of slope +2); ■, $\tilde{k}_1 = 0$. (*b*) pH range down to 0: △, $\tilde{k}_1 = 0.01$ (the continuous line is of slope +1); ●, $\tilde{k}_1 = 0.001$ (the continuous line is of slope +1); ○, $\tilde{k}_1 = 0.0001$; ■, $\tilde{k}_1 = 0$ (the continuous line is slope +2).

not be possible to collect data at pH values low enough to observe deviations in the logarithmic plots because of enzyme instability, particularly if $\tilde{k}_1$ is not less than approx. 1% of $\tilde{k}_2$.

Catalytic activity and thiol reactivity characteristics of papain assessed in terms of overlapping protonic dissociations

Scheme 3 is a specific version of the general model of Scheme 2 as it might relate to the active centre of papain. In the context of the present paper, the pH-dependence data reported by Polgár & Halász (1978) are particularly interesting and could be nicely interpreted in terms of the variation in $pK_{app.}$ predicted by the simulation study discussed above. Thus the values of the first measured pK_a value of papain apparently characteristic of a sigmoidal profile, as determined by various alkylation and acylation reactions, range over about 1 unit. This is the limit of variability predicted by the simulation study of the model of Scheme 2, and this maximum variation would predict some reactions in which $\tilde{k}_1$ (of Schemes 2 and 3) is very small relative to $\tilde{k}_2$ and some reactions in which the values of these rate constants (equivalent to pH-independent values of $k_{cat.}/K_m$ for the acylations) are closely similar. Evidence has accumulated that reactions of the papain thiol group might be modulated by several ionizing groups (see Jarvis & Brocklehurst, 1982, and references cited therein). The remarkable variation in $pK_{app.}$ shown by the careful studies by Polgár & Halász (1978) suggests that the reactions of the simple neutral alkylating agents methyl bromoacetate and ethyl bromoacetate ($pK_{app.}$ 3.25) are not influenced by the ionization state of groups additional to the S^-/ImH^+ ion-pair and that $\tilde{k}_1$ and $\tilde{k}_2$ (of Schemes 2 and 3) are not very different from each other. This would provide a value of $pK_{app.}$ at the lower end of the 1-unit range of variation.

This lower limit of $pK_{app.}$ is closely similar to the pK_a (3.3) of the thiol group of cysteine-25 when it forms the S^-/ImH^+ ion-pair deduced from proton-n.m.r. studies (Lewis *et al.*, 1981).



Scheme 3. *Kinetic model for the reaction of the thiol group of papain in two protonic states in acidic media*
SH is part of the side chain of cysteine-25; ImH^+ is the imidazolium ion of histidine-159; XH is part of the side chain of another amino acid residue, which might be aspartic acid-158 or another residue remote from the active-centre region; K_I and K_{II} are molecular (macroscopic) acid dissociation constants characteristic of the ionization of (I) and of [(II) + (III) + (IV)] respectively; $\tilde{k}_1$ and $\tilde{k}_2$ are the pH-independent rate constants characteristic of the reaction of the two reactive protonic states [(II) + (III) + (IV)] and [(V) + (VI)] respectively. The reactive components of these states are considered to be those containing thiolate anion [i.e. (IV) and (VI)]. pK_I and pK_{II} are in the range approx. 3–5. The further ionization of (V) and (VI) with pK_{III} = 8–9 to produce the additional nucleophilic state ($^-$X–Im–S$^-$) is not considered. Free protons are not shown.

By contrast, acylation of the thiolate anion of the ion-pair of papain by substrates ($pK_{app.}$ 4.0–4.3) would appear to be more efficient when an additional group (pK_a approx. 4) is proton-deficient, i.e. $\tilde{k}_1$ (Schemes 2 and 3) is a small fraction of $\tilde{k}_2$.

It is not surprising that simple alkylation reactions of the thiolate anion might have less stringent geometrical requirements than the acylation process of the catalytic act and that the geometry of the catalytic site at various stages of the acylation process might be controlled by additional ionizations. The two-state UP/DOWN mechanism (Angelides & Fink, 1978, 1979*a*,*b*); Brocklehurst & Malthouse, 1978; Brocklehurst *et al.*, 1979*a*,*b*) contributes one specific suggestion of how such modulation might occur, but the general concept depicted in Schemes 2 and 3 is not restricted to this particular mechanism nor to the carboxy group of aspartic acid-158 as the modulating group.

In view of the accuracy and extent of the kinetic data (in pH regions when the enzyme is conformationally unstable) needed to reliably establish a slope of +2 in logarithmic plots of data such as those shown in Figs. 1 and 2, it seems that marked variation in values of $pK_{app.}$ might be a more convincing way in some cases of deducing the kinetic influence of additional ionizing groups.

In the data reported by Polgár & Halász (1978) the reaction of chloroacetate ($pK_{app.}$ 3.6) occupies an intermediate position in the 1-unit range of values of $pK_{app.}$. This might be predicted in terms of Schemes 2 and 3 in that the bell-shaped pH–*k* profile for the reaction of this anionic alkylating agent suggests a requirement for association of the carboxylate anion of the reagent with the imidazolium ion of the ion-pair simultaneously with reaction of the thiolate ion at the electrophilic carbon atom. This degree of organization might be some way towards that required in the acylation step of a catalysis.

One apparently anomalous result in the data presented by Polgár & Halász (1978) is the alkylation reaction by bromoacetamide. The value of $pK_{app.}$ for this reaction (4.0) might have been predicted to be about 3.3, as was found for the bromoacetate esters. In terms of Schemes 2 and 3 this result suggests a degree of organization of the active centre of papain similar to that found with

substrates. The reason for this high value of $pK_{app.}$ remains to be established. The only structural difference between this reagent and the esters is the presence of the amide group. This might be able to engage in a hydrogen-bonding interaction in the active-centre cleft, and such hydrogen-bonding interactions (see Lowe, 1976) might assist in organizing catalytic-site geometry. If this is the case, this type of interaction would seem to be linked with the ionization of the modulating group deduced above.

Conclusions

Overlapping protonic dissociations can provide both pH–*k* profiles that are probably indistinguishable in practice from a simple sigmoidal shape and free-enzyme $pK_{app.}$ values that are subject to variability over a range of about 1 unit. Such variation is a good indication of the involvement of additional kinetically influential proton-dissociating groups, and is probably more reliable as a criterion for the existence of such groups than deviations from slopes of +1 in logarithmic plots, particularly at low pH. Conformational selectivity cannot result in variation of free-enzyme $pK_{app.}$ values. The efficient acylation of papain by substrates requires a proton-deficient group additional to the S^-/ImH^+ ion-pair associated with a molecular pK_a value of about 4. The state of ionization of this group does not affect the reaction of S^-/ImH^+ with some simple alkylating agents, but the additional group (pK_a approx. 4) required to be dissociated in acylation might be the same group that affects the reactions of the papain thiol group with 2,2′-dipyridyl disulphide and with 4-chloro-7-nitrobenzofurazan (see Brocklehurst, 1982; Shipton *et al.*, 1976).

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